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AD-A248 892

ON PAGE

Form Approved
OMB No. 0704-0188

AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	February 4, 1992	

4. TITLE AND SUBTITLE

Translational Regulation of Cloned Genes

5. FUNDING NUMBERS

6. AUTHOR(S)

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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U. S. Army Research Office
P. O. Box 12211
Research Triangle Park, NC 27709-2211

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

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12a. DISTRIBUTION / AVAILABILITY STATEMENT

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12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 words)*

The factors controlling translational efficiency of mRNA in yeast were studied to aid in design of more efficient vectors for producing heterologous proteins in yeast. A plasmid was constructed with heterologous gene production driven by the yeast CUP1 promoter and the killer virus M segment translation initiation signal. Although capped RNAs were more efficiently translated in vitro than uncapped RNAs of the same sequence, changes in the 5' untranslated leader rendered uncapped RNA as translatable as wild-type capped RNA. This result may make cytoplasmic RNA-based cloning vectors feasible in yeast.

14. SUBJECT TERMS

yeast, recombinant DNA gene expression, translation, transciption, polymerase chain reaction, mRNA cap

15. NUMBER OF PAGES

29

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
UNCLASSIFIED	UNCLASSIFIED	UNCLASSIFIED	UL

TRANSLATIONAL REGULATION OF CLONED GENES

FINAL REPORT

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FEBRUARY 4, 1992

U.S. ARMY RESEARCH OFFICE

CONTRACT/GRANT NUMBER DAAL03-88-K-0196
PROPOSAL NUMBER 24931 - LS

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1. FOREWORD

The yeast *Saccharomyces cerevisiae* is widely used as a host for recombinant DNA plasmids expressing heterologous genes, for the purpose of molecular genetic studies and for production of the products of these genes for medical and industrial applications. Although the factors determining the efficiency of gene expression at the level of transcription have been extensively studied in yeast and other organisms, efforts at expressing cloned genes have been hampered by the unpredictability of translation of various genetically engineered mRNA molecules. Therefore, starting with the known sequence of the m transcript derived from the M double-stranded (ds) RNA segment of killer virus of yeast, we set out to determine the effect of leader sequence and secondary structure on the translatability of mRNA in yeast. This work will be applicable to the design of better DNA-based expression vectors for production of heterologous proteins in yeast, and potentially could find further application in the design of vectors based on the RNA genome of killer virus.

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3. *LIST OF FIGURES*

4. BODY OF REPORT

A. STATEMENT OF PROBLEM

The major aim of this work was to determine the structural basis of the differences in the efficiency translation observed between different mRNA species. Various heterologous genes cloned in yeast have been found to differ in their efficiency of translation, and this difference is only partly explained by the presence of poorly utilized codons in the structural genes (1, 2). In eukaryotic cells, conformity to a consensus sequence around the first AUG initiation codon following the terminal cap structure correlates with efficiency of translation initiation, although the effect of the sequence in this region on translation has been limited to a two-fold effect in yeast (reviewed in 3). Secondary structure in the mRNA region 5' to the initiating AUG has been shown to inhibit translation *in vivo* of capped nuclear transcripts in yeast (4). This project was designed to determine the optimal translation initiation signal of yeast *in vitro* and *in vivo*, so that this sequence could be used in cloning vectors to optimize translational initiation in yeast. This determination was made both using capped and uncapped mRNAs, since it seemed possible that the initiation mechanism on these two types of templates might differ. By using *in vitro* transcribed RNA from cDNA clones as templates for translation, the translatability of capped and uncapped RNA of each sequence analyzed could be directly measured. This *in vitro* approach has the advantage of allowing us to compare capped and uncapped RNAs of the same sequence, rather than chemically de-capping biological RNA extracted from cells. A new vector for expression of cloned genes in yeast was the goal of this project, with transcription driven by the inducible *CUP1* promoter of yeast and with translation driven by the optimal translation-initiation signal, to be determined.

During the course of this work, the reports that both yeast (5) and *Giardia lamblia* (6) could be transfected with RNA derived from RNA viruses suggested that RNA cloning vectors might also be designed for heterologous gene expression in yeast. Since the RNA transcripts of the dsRNA genomes of yeast viruses appear to be uncapped (7), determination of the optimal translation signal for uncapped mRNA would be essential for this project, which is planned as a future goal of this laboratory.

B. SUMMARY OF MOST IMPORTANT RESULTS

1. Biology of killer virus

Killer virus of yeast has long been known to be temperature sensitive, so that strains of yeast persistently infected with the virus can be cured by growth at elevated temperatures (8). Experiments in this laboratory (9) and elsewhere (10) indicated that there was great variability between different infected strains in their heat curability. We found that this difference was genetically correlated with the allelic form of the L-A dsRNA segment of the virus (9); the allele L-A-HN confers heat curability, while the allele L-A-H confers heat incurability to the M dsRNA satellite infecting the same cell. The M dsRNA satellite encodes the toxin and resistance substance which give the killer virus its name. The replication of both dsRNA segments depend on the capsid and capsid/RNA polymerase fusion proteins encoded by L-A dsRNA (11-13), and

presumably allelic differences in the genes encoding one of these proteins are responsible for the observed difference in curability. We also observed that diploid host cells are more readily heat-cured of the virus than are haploids (9). This interaction of ploidy and mating type with viral functions is also reflected in the cytoplasmically inherited diploid-dependent [kil-d] mutants, which express viral phenotypes normally in diploid cells but are defective in viral replication and expression in haploid hosts (14). In the course of studies on these mutants we discovered that the diploid-dependent mutation does not reside on the M dsRNA segment (9), as previously (14) claimed. The complexity of the interaction of killer virus with the host cell continues to be studied in this laboratory.

2. Killer virus transcription initiation *in vitro*

Killer virions isolated from infected *Saccharomyces cerevisiae* cells contain an RNA polymerase activity which catalyzes the transcription of positive polarity RNAs from the L-A and M dsRNA genomic segments of the virus. The RNA polymerase can initiate transcription *in vitro* with gamma-thio-GTP, whose thiophosphate group is found on the 5′ terminus of transcripts. Transcripts produced *in vitro* in the presence of ^{7}mGpppG are significantly more active as translational templates than are transcripts produced in it absence. However, unlike *Escherichia coli* RNA polymerase transcripts from viral cDNA made in the presence of ^{7}mGpppG, transcripts produced by viral RNA polymerase in the presence of ^{7}mGpppG fail to bind antibody against ^{7}mG. The increased translational activity of the viral transcripts made in the presence of ^{7}mGpppG suggests that these transcripts can be capped by priming with the cap analog; the failure of these transcripts to bind the antibody suggests that either the cap is somehow modified in the process or otherwise made unavailable to the antibody, unlike a cap-primed cDNA-derived transcript of the same sequence (15).

3. Coupling of viral transcription with translation

Since killer virus persists in the cytoplasm of infected cells (16), transcription and translation of its apparently uncapped transcripts both occur in the same cellular compartment. Virions purified from infected cells can be added to cell-free translational extracts (17) of uninfected cells, resulting in a reaction in which viral transcription and translation are coupled. In this reaction, which has an optimal temperature of 15°C, nucleotides are incorporated into full-length transcripts of the M and L-A dsRNA segments, with lower levels of incorporation into genomic RNA. In addition, incorporation of nucleotides is observed into a smaller RNA species showing no sequence relatedness to M or L-A (18).

4. Omega translational enhancer of tobacco mosaic virus

In tobacco mosaic virus (TMV) mRNA, a 5′ terminal untranslated leader sequence, denoted omega, has been observed to function as a non-specific translational enhancer, apparently by providing an alternative site for translation initiation, analogous in function to the function of cap structures in eukaryotes and Shine-Dalgarno sequences in many prokaryotes (19-21). Utilizing synthetic omega DNA made in this laboratory as starting material, we cloned omega 5′ to the preprotoxin cDNA in a DNA plasmid in yeast, but did not

show increased expression of killer toxin in cells transformed with this plasmid (22). A series of mutants in the omega sequence were also generated (22).

5. Construction of yeast expression vectors

We followed the steps outlined in the original proposal to generate a plasmid for testing expression of heterologous genes in yeast. The original version of this vector contains a chimeric M dsRNA-derived cDNA linked in-frame to the *lacZ* gene of *E. coli*, under the transcriptional control of the copper-inducible *CUP1* (metallothionein) promoter of yeast. Modified versions of the vector with altered 5' untranslated leaders can be made from the cDNA constructs described in the next section. Since the end of this support period, we are continuing to refine our vector design for improved usefulness and increased expression levels. As discussed above, the availability of transformation methods for RNA (5-6) has suggested that we can extend this work to the development of RNA vectors.

6. Optimization of translation initiation signals

In order to determine the optimal translation initiation signal for mRNA in yeast, various mutant mRNAs were tested as translational templates in a yeast cell-free translation system developed in this laboratory (17). We have also developed a method of generating mutants *in vitro*, by "mutagenic" polymerase chain reaction (PCR), followed by *in vitro* mRNA synthesis catalyzed by bacteriophage SP6 DNA-dependent RNA polymerase. Thus mutants are rapidly generated and phenotypically screened *in vitro*.

For these experiments, we started with a plasmid containing the complete cDNA derived from killer virus of yeast M dsRNA, except that the polyA·polyU tract separating the M_1-1 (preprotoxin-coding) region from the non-coding M_1-2 region was replaced by a convenient DNA linker. In order to produce a DNA template suitable for generation of full-length RNA copies identical to the viral m transcript from this cloned M cDNA, the plasmid was first subjected to PCR amplification utilizing one primer collinear with the 5' terminus of the positive M dsRNA strand and containing a 5' extension with the sequence of the bacteriophage SP6 promoter upstream sequences and a second primer collinear with the 5' terminus of the negative strand of M dsRNA. The resulting PCR product was utilized as a template for SP6 DNA-dependent RNA polymerase to generate transcripts which were confirmed by direct RNA automated sequence analysis to have the same 5'-terminal sequence as viral m transcript or positive strands of viral genomic M dsRNA. This PCR-derived m transcript, upon addition to a yeast cell-free translation extract, stimulated incorporation of ^{35}S-methionine into the 32 kd. preprotoxin encoded by the only open reading frame on M dsRNA. This translation reaction could be quantitated by incorporation of ^{35}S into TCA-precipitable material or into a 32 kd band on SDS-PAGE, as determined by cutting out the band for liquid scintillation counting or by phosphorimaging with computerized image quantitation (Molecular Dynamics Phospho-Imager). When PCR-product transcription by SP6 RNA polymerase was performed in the presence of excess 7mGpppG, capped RNA transcripts were generated, which showed the same yield and electrophoretic mobility as RNA transcripts made in the absence of caps. Upon translation, the capped transcripts showed 8-12 times the specific template activity of uncapped transcripts, based on either the TCA or gel assays

for translation. All assays were corrected using blank reactions to which no template RNA was added so RNA-stimulated translation was measured in all reactions. The wild-type m transcript contains a 5′ terminal stem-and-loop structure involving the first 64 nucleotides, as shown in Fig. 1. This structure includes atypical A-G base pairs (at nucleotides 14 and 22) and A-C base pairs (at nucleotides 20 and 24); both of these base pairs have been reported to occur in the context of double-helical regions of RNA or synthetic oligonucleotides (23-24), and the structure shown agrees with nuclease S1 sensitivity mapping of single and double-stranded regions of this hairpin (7, 25). Fig. 1 also shows the free energy predicted for this region based on the Wisconsin GCG "fold" program (which does not consider atypical A-C and A-G base pairs) and utilizing the free energies as predicted by Salser (26) but considering such atypical base pairs as occurring and not contributing any free energy to the structure shown.

Fig. 1 also indicates the theoretical stability of base-pairing of this mRNA to the 3′ terminal regions of yeast 5.8S and 18S rRNAs. This complementarity was previously noted based upon sequence comparisons (7, 25), but its physiological significance was unknown until the present mutagenesis studies. In all figures the initiating AUG codon is underlined. Asterisks (*) mark nucleotides which differ from the wild-type sequence shown in Fig. 1.

Mutant PCR products were generated as described above for the wild-type product, except that the 5′ positive strand primer was a "mutagenic" primer, containing the SP6 promoter (17 bases), the mutant 5′ terminal sequence and a 3′-terminal G or C residue. Mutant PCR products were generated utilizing either Taq DNA polymerase (Perking Elmer Cetus or Promega Biotech) or DNA polymerase from *Thermus thermophilus* (Dupont or Amersham), the latter showing greater ability to utilize primers containing regions of stable internal secondary structure. Mutant PCR products were all the same size as the wild-type products as measured by agarose gel electrophoresis. Mutant PCR products were transcribed to generate mutant SP6 polymerase transcripts, which were then tested as translational templates, and compared with the wild-type transcript run in a parallel reaction. All transcripts were tested at the same RNA concentration, as estimated by quantitation of an ethidium bromide stained agarose electrophoretic gel. For many mutants the 5′ terminal sequence of the RNA was directly confirmed, and for all the DNA sequence of the PCR product encoding this region of the transcript was determined.

As is shown in Fig. 2, the mutant RNA 5POSMALT1, A14→U, which eliminates the initiating AUG of the preprotoxin gene virtually eliminates translation of capped or uncapped RNA (by either assay). This result confirms that the mutagenesis technique can generate altered mRNA and that the first AUG of the m transcript is the translation initiation site for capped or uncapped transcripts.

Fig. 3 illustrates the mutant transcript 5POSMALT2, which has two base charges at positions 13 and 19 to reduce the theoretical free energy of 5.8S rRNA binding, calculated according to Salser (26), from -13.5 to -7.8 kcal. This change results in no decrease (actually a modest increase) in the translational template activity of capped or uncapped m transcript. Fig. 4 indicates that a mutant with changes in nucleotides 20-22 to increase 5.8S rRNA binding (mutant 5POSMALT3) stability (free energy -22.0 kcal.) has about the same template activity as wild-type RNA. Therefore, it appears that the theoretical stability

of 5.8S rRNA base-pairing with the initiation region of m transcript is not a major factor in determining translational template activity.

We have proposed (25) that the 5' terminal secondary structure of m transcript is a factor negatively affecting its translational template activity, as has been reported for non-viral mRNAs in yeast (4). Fig. 5 shows our results for the mutant transcript 5POSMALT5, which has nucleotide changes which virtually completely destabilize the 5' secondary structure of m transcript without changing the sequence of the encoded protein. This change results in a 2.4-3.2-fold increase in template activity of capped or uncapped transcripts, confirming the inhibitory effect of 5'-terminal secondary structure on translation. This conclusion is also confirmed by Fig. 6 which shows the results of translation of 5POSMALT7, a mutant with increased stability of its 64-nucleotide stem-and-loop structure. The data indicate that this mutant results in a transcript with virtually no template activity. Note that our modification of Salser's (26) free energy calculation indicates this structure to be much more stable than does the Wisconsin-GCG fold program which does not allow atypical A-C and A-G base pairs to be considered.

Figure 7 shows the structure of the transcripts of 5POSMALT13, which has the tightened secondary structure as in 5POSMALT7 (Fig 6) for bases 10-28, but which has reduced secondary structure stability for bases 1-9. The data indicate that this structure partially restores the translational template activity of capped RNA with this sequence, and uncapped 5POSMALT13 RNA is even more active than wild-type uncapped RNA. This result suggests that despite several additional base pairs just upstream from the initiating AUG, 5POSMALT13 is an active translational template due to its unpaired 5' terminal 9 bases. This requirement for an unpaired 5' terminal region seems to be even stronger for uncapped than capped RNA since the changes in 5POSMALT13 are more stimulatory to translation of uncapped RNA. Also note that 5POSMALT13 lacks the G at position 11; this change may also contribute to uncapped RNA template activity (see below).

Various RNA viruses of eukaryotic cells face the problem of expressing their uncapped messages in a cytoplasm which prefers capped RNA templates for translation. Tobacco mosaic virus uncapped mRNA may be expressed due to a 5'-terminal sequence of about 70 nucleotides, denoted omega, which is A, U-rich, G-poor and appears to serve as a site for cap-independent translation initiation (19-21). The length of omega varies somewhat among the tobacco mosaic virus strains, but is much longer than the 13 nucleotide leader of m transcript of killer virus of yeast. Although uncapped RNA is generally not very active as a translational template, the results in Fig. 7 suggest that there may be modifications of this leader which would enhance its template activity, perhaps by a mechanism similar to translational enhancement by omega.

Fig. 8 shows our results for 5POSMALT9, which yields a transcript with two base changes destroying the initiating AUG and changing codon 2 to AUG, thus moving the initiating AUG 3 nucleotides distally. This change caused a 2.4-3 fold increase in translatability of capped RNA and a 3.1-3.5 fold increase for uncapped RNA. Fig. 9 shows the results for 5POSMALT8, which is only altered by changing G11 to U. Here we again see a difference in effect on translation of capped and uncapped RNA; this change causes a 50% inhibition of translation of

capped RNA but has no effect on uncapped RNA. When this G11 to U change was added to the loosening of secondary structure generated in 5POSMALT5 (Fig 5), 5POSMALT11 was produced (Fig. 10). The capped transcript of 5POSMALT11 was about as active as 5POSMALT5, but the uncapped transcript showed even greater translational template activity, over 5-fold more than the wild-type uncapped sequence.

Fig. 11 (5POSMALT10) shows the mutant resulting in the most stimulation of uncapped RNA which we have yet identified. This mutant has its AUG shifted three nucleotides distally and eliminates four potential base-pairing sites just before the new AUG. These changes significantly stimulate translation of capped transcripts, but result in a 12.5-13.4 fold stimulation of translation of uncapped RNA, thus making this uncapped mutant RNA as active a translation template as capped wild-type RNA. This result is intriguing, because as reviewed by Kozak (3), various attempts to alter the leader sequences of nuclear gene transcripts in yeast have only yielded up to two-fold changes in translatability of their capped transcripts. Apparently the translatability of uncapped m killer virus transcripts in the yeast cell-free system can be markedly improved by small changes in structure, which generate a short omega-like sequence acting as a translational enhancer. The *in vivo* expression of similar DNA mutants is now being tested using the heterologous *lacz* gene in DNA constructs.

Recent reports indicating the transfectability of *Giardia lamblia* by viral transcripts (6) or of yeast by killer virus RNA (5) raises the possibility that killer virus related RNAs derived from cDNA might be developed as transfection vectors for heterologous gene expression. Optimization of translation initiation signals on the uncapped transcripts by viral transfection vectors should improve their usefulness for this purpose. We are currently developing methods for transfection of yeast cells with cDNA-derived RNA. The development of such an RNA-virus based cloning system is a major focus of attention in our laboratory.

C. LIST OF ALL PUBLICATIONS AND TECHNICAL REPORTS

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Lee Ann Weinstein, Ph.D., awarded 1991

5. REPORT OF INVENTIONS None

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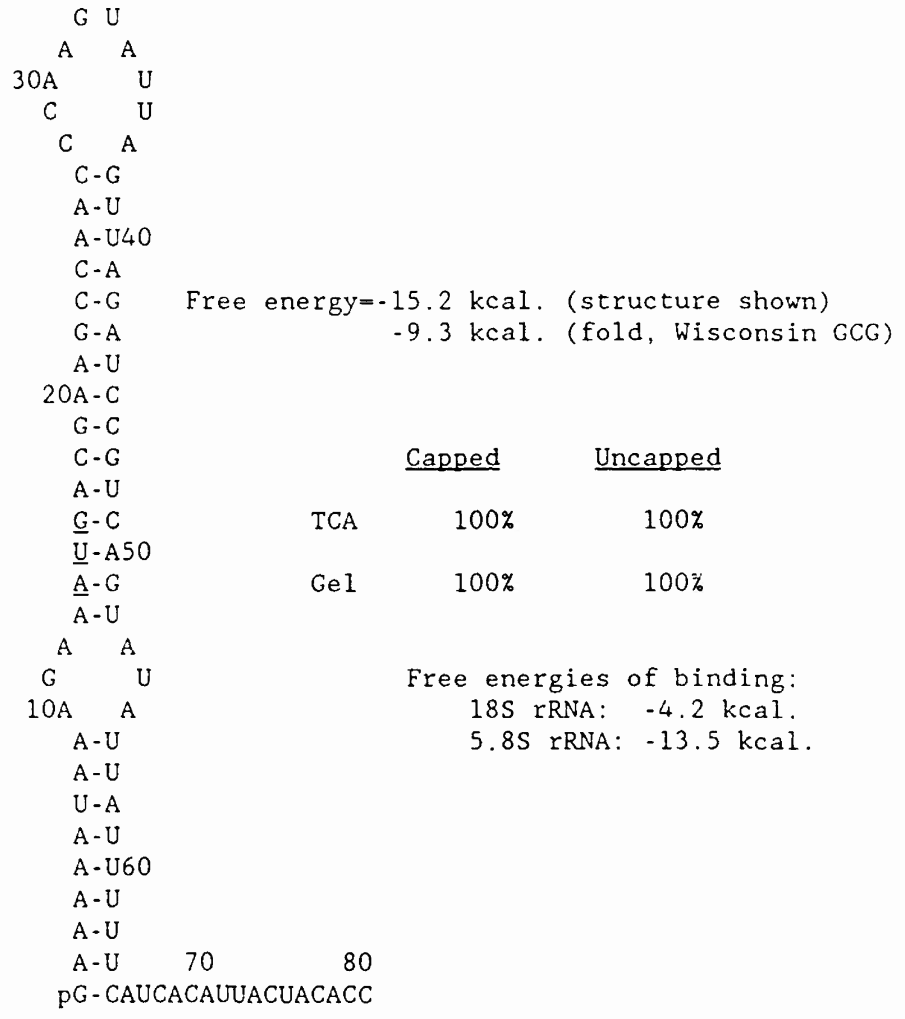
7. FIGURES

Figure 1 Structure of wild-type m transcript

WILD-TYPE (5POSM)



Free energy=-15.2 kcal. (structure shown)
-9.3 kcal. (fold, Wisconsin GCG)

		Capped	Uncapped
TCA		100%	100%
Gel		100%	100%

Free energies of binding:
18S rRNA: -4.2 kcal.
5.8S rRNA: -13.5 kcal.

Figure 2 Mutant RNA lacking initiation AUG

AUG ABSENT (5POSMALT1)

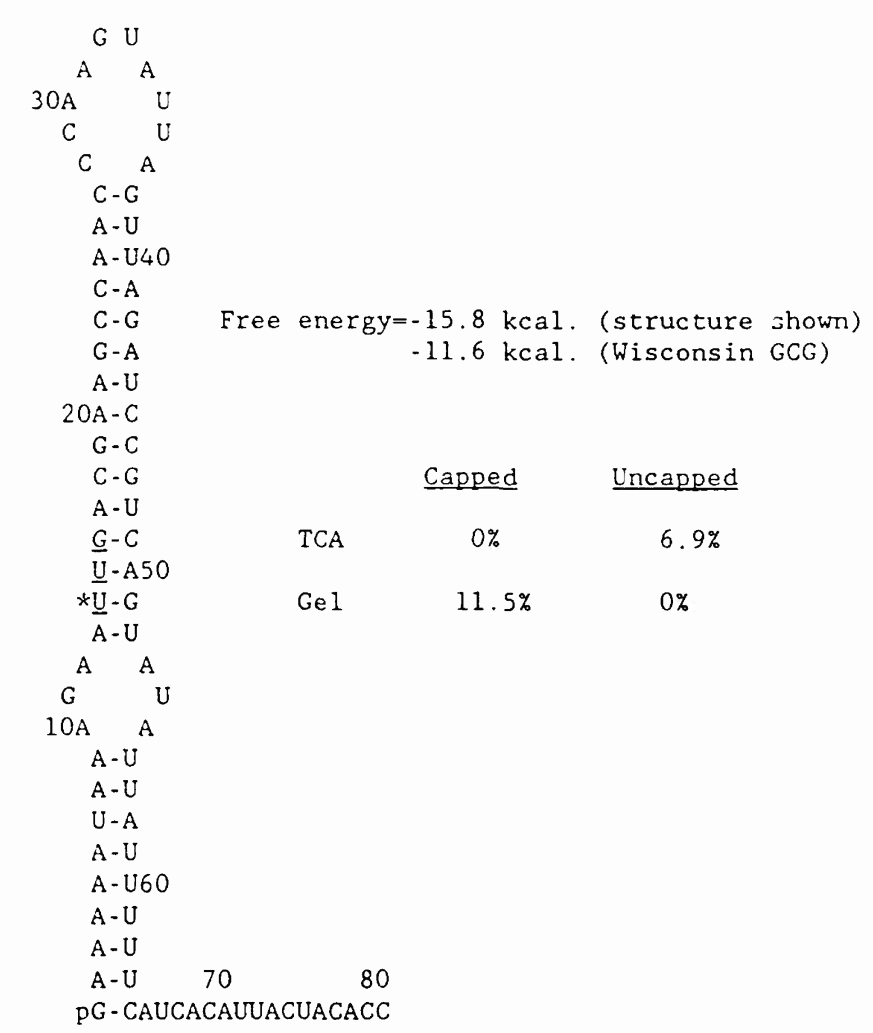
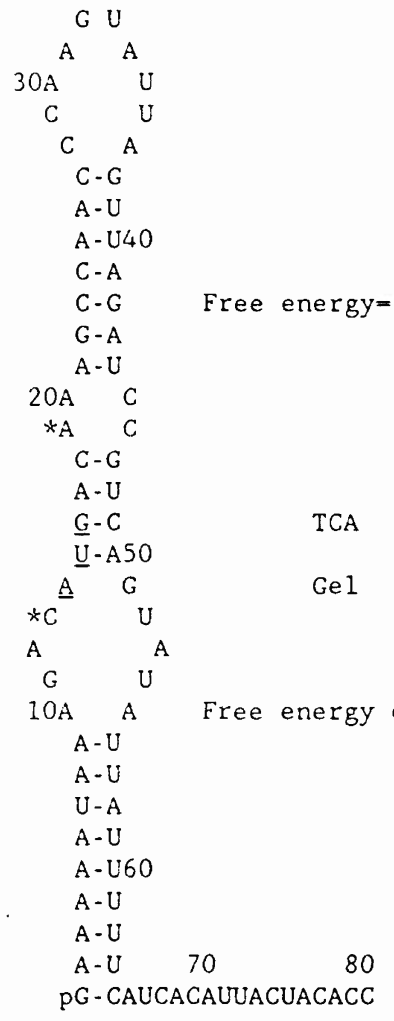


Figure 3 Mutant with weakened 5.8S rRNA binding

WEAKEN 5.8S rRNA BINDING (5POSMALT2)



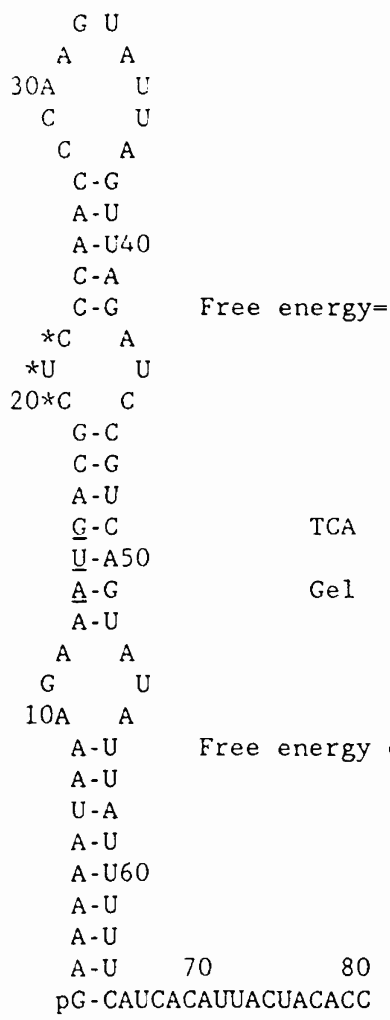
Free energy=-9.5 kcal. (structure shown)
-9.1 kcal. (fold, Wisconsin GCG)

	Capped	Uncapped
TCA	112%	134%
Gel	144%	263%

Free energy of 5.8S rRNA binding=-7.8 kcal.

Figure 4 Mutant with strengthened 5.8S rRNA binding

INCREASE STABILITY OF 5.8S rRNA BINDING, CODING CHANGE LYS3->LEU (5POSMALT3)



Free energy=-12.7 kcal. (structure shown)
-11.8 kcal. (fold, Wisconsin GCG)

	Capped	Uncapped
TCA	86%	82%
Gel	105%	133%

Free energy of 5.8S rRNA binding=-22.0 kcal.

Figure 5 Mutant with decreased hairpin stability

WEAKEN HAIRPIN (5POSMALT5)

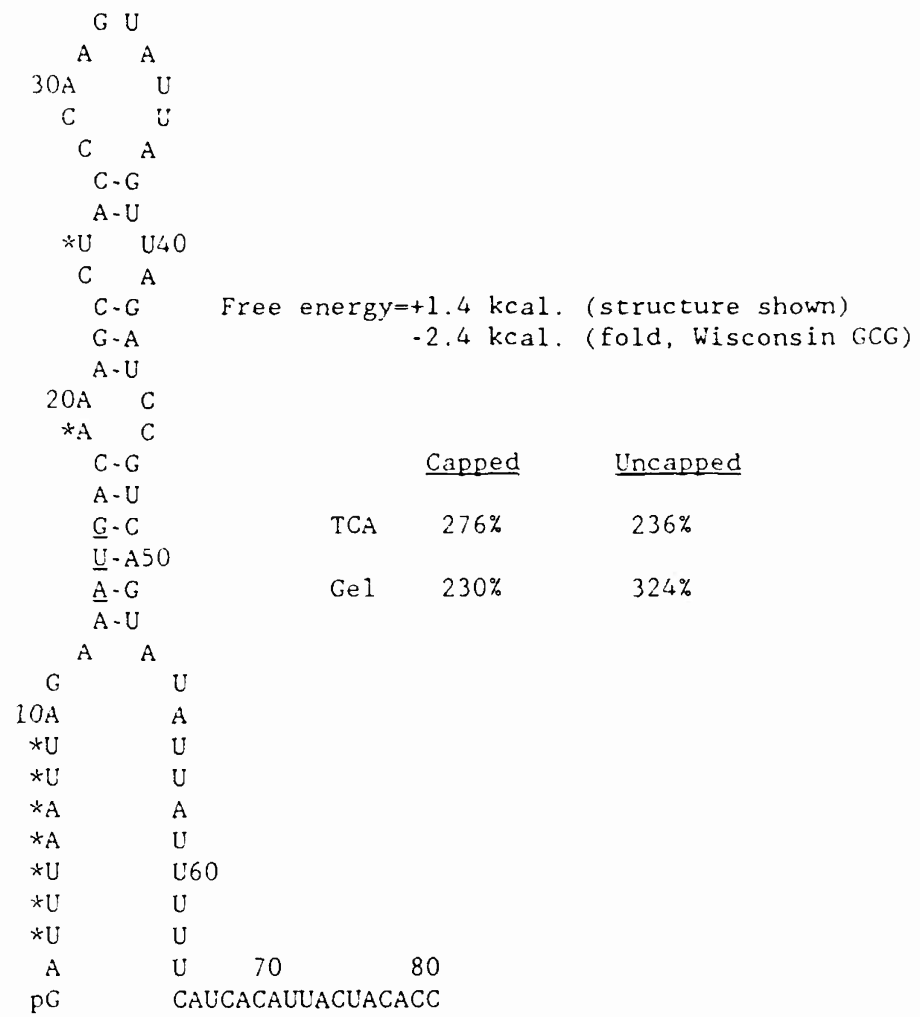
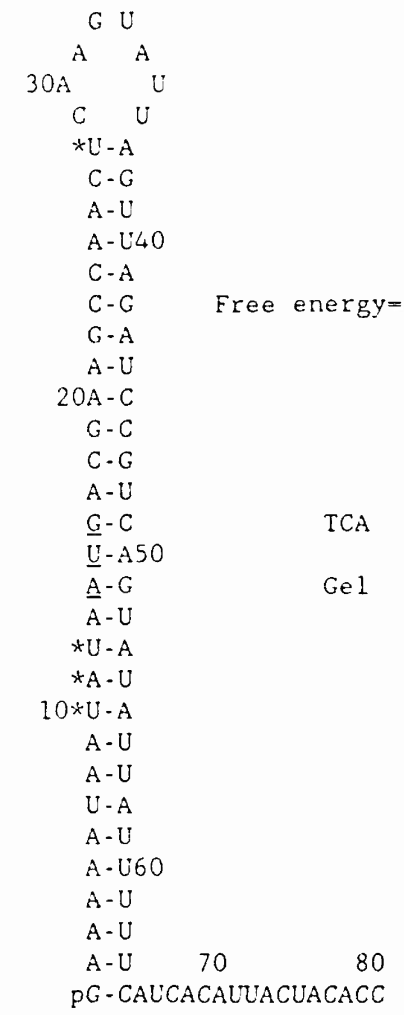


Figure 6 Mutant with increased hairpin stability

STRENGTHEN HAIRPIN (5POSMALT7)

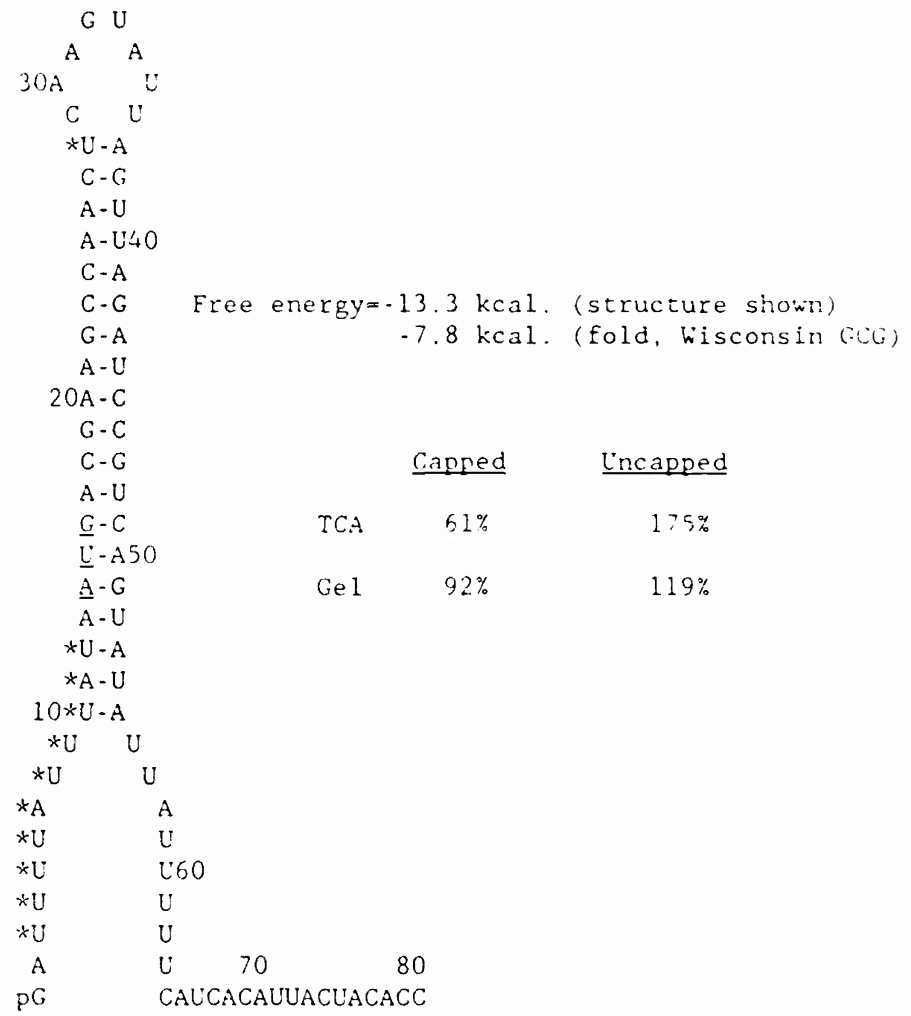


Free energy=-26.8 kcal. (structure shown)
-17.3 kcal. (fold, Wisconsin GCG)

		Capped	Uncapped
TCA		0%	5%
Gel		14%	13%

Figure 7 Mutant with hairpin destabilized at base, stabilized near loop

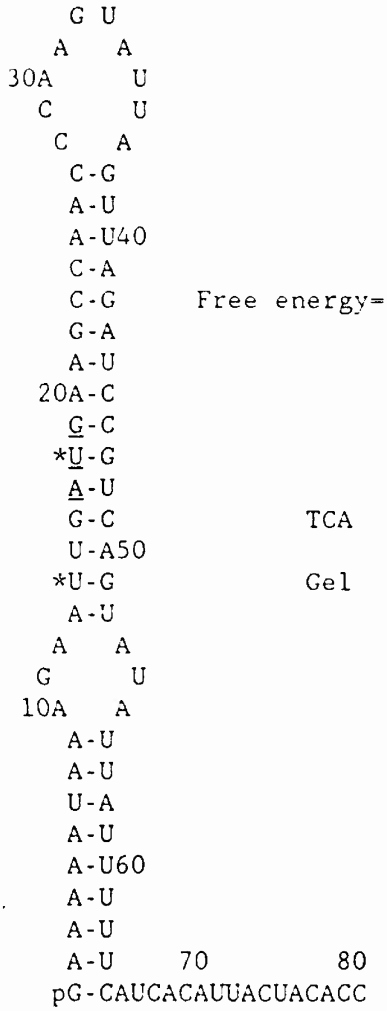
WEAKEN HAIRPIN AT BASE, CONSERVING CHANGES IN 5POSMALT7 (5POSMALT13)



	Capped	Uncapped
TCA	61%	175%
Gel	92%	119%

Figure 8 Mutant with leader lengthened by 3 nucleotides

MOVE AUG ONE CODON DISTAL, THR2->MET (5POSMALT9)

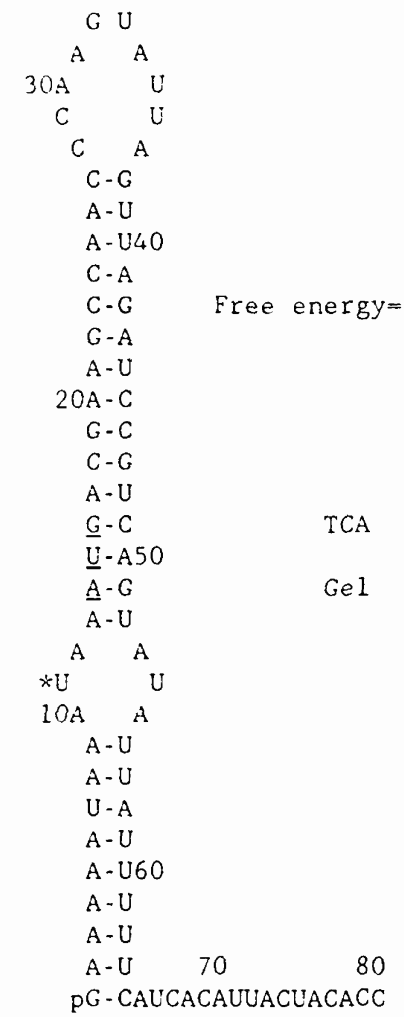


Free energy=-12.3 kcal. (structure shown)
-9.7 kcal. (fold, Wisconsin GCG)

	Capped	Uncapped
TCA	296%	346%
Gel	237%	311%

Figure 9 Mutant lacking G at -3 position

G11->U (5POSMALT8)

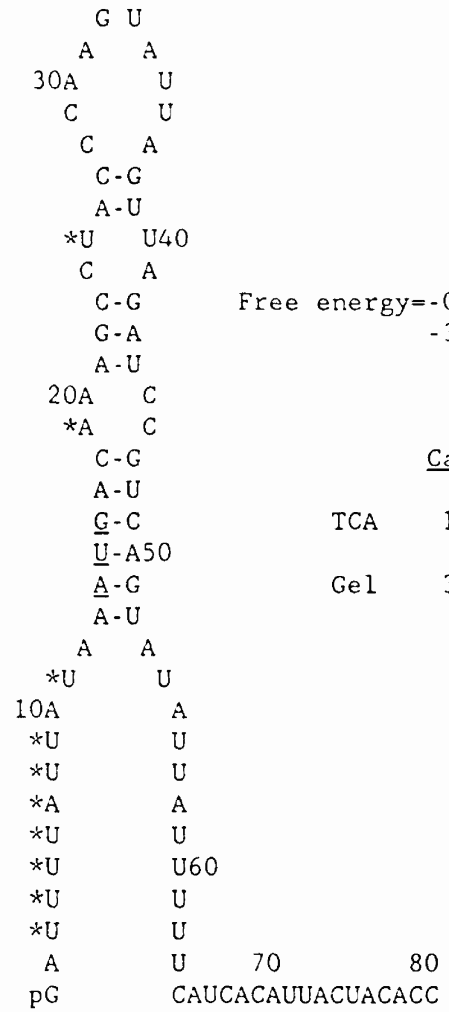


Free energy=-15.2 kcal. (structure shown)
-11.4 kcal. (fold, Wisconsin GCG)

	Capped	Uncapped
TCA	50%	100%
Gel	54%	123%

Figure 10 Mutant with weakened hairpin and lacking G at -3

WEAKEN HAIRPIN AS IN 5POSMALT5 & G11->U (5POSMALT11)

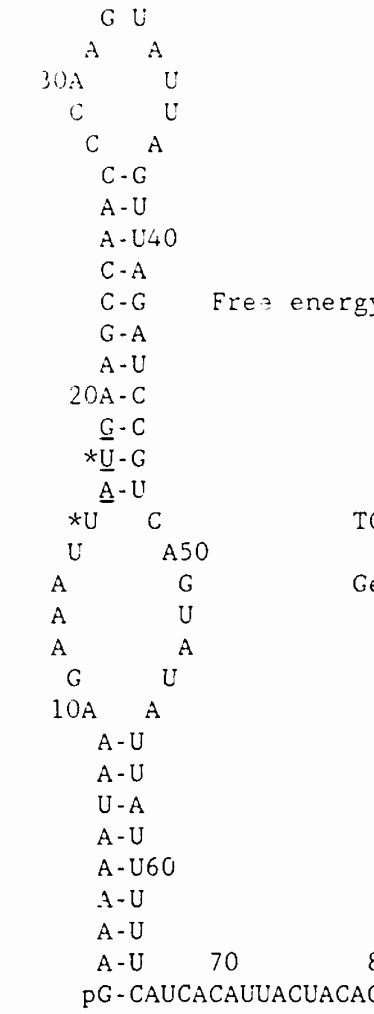


Free energy=-0.7 kcal. (structure shown)
-3.9 kcal. (fold, Wisconsin GCG)

	Capped	Uncapped
TCA	150%	520%
Gel	300%	542%

Figure 11 Mutant with optimal translatability in uncapped form

MOVE AUG ONE CODON DISTAL, WEAKEN HAIRPIN, THR2->MET (5POSMALT10)



Free energy=-6.8 kcal. (structure shown)
-4.8 kcal. (fold, Wisconsin GCG)

	Capped	Uncapped
TCA	350%	1337%
Gel	502%	1248%

8. APPENDIX

The following abstracts, not previous transmitted, are included in this section:

Lee Ann Weinstein, Florence Capaldo-Kimball, Michael J. Leibowitz, Deborah Abel, Sandra Rosborough, and Euton Laing, Effect of temperature on replication and expression of yeast killer virus. Abstracts of 90th Annual Meeting of the American Society for Microbiology, Abstract T17 (1990).

Lee Ann Weinstein, Florence Capaldo-Kimball, Michael J. Leibowitz, Deborah Abel, Sandra Rosborough, and Euton Laing, Host and viral factors affect the heat curability of killer virus of yeast. Abstracts of the Theobald Smith Society (New Jersey Branch, A.S.M.) Annual Meeting-in-Miniature, p. 17 (1990).

Michael J. Leibowitz, Denise E. Georgopoulos, and Lee Ann Weinstein, Genetics of heat curability of the killer virus of *Saccharomyces cerevisiae*; *in vitro* mutagenesis and phenotype testing of cDNA clones. Abstracts of EMBO International Workshop on Viruses of Fungi and Simple Eukaryotes, Mallorca, Spain, p. 8 (1991)

Effect of Temperature on Replication and Expression of Yeast Killer Virus. L. WEINSTEIN*, F. CAPALDO-KIMBALL, M.J. LEIBOWITZ, D. ABEL, S. ROSBOROUGH, E. LAING, UMDNJ-Robert Wood Johnson Medical School, Piscataway, N.J.

Killer virus is a cytoplasmically-inherited virus of Saccharomyces cerevisiae. Its genome consists of two separately encapsidated segments of double-stranded (ds) RNA. The different strains of killer virus are denoted K1, K2, etc. We are studying the heat-induced loss of killer virus from various host-virus combinations to optimize a temperature-sensitive (ts) model for studying the replication and expression of dsRNA viruses in eukaryotic hosts. Virus-infected cells were grown for 100 hours at 37°C. Curing was tested at varying times by killer phenotype assays on petri dishes, and by polyacrylamide gel electrophoretic analysis of dsRNA. We have determined that (1) K1 virus is more curable than K2, (2) viruses of either type are cured more readily in diploid than in haploid cells, and (3) grande yeast cells harboring the virus are cured of the virus sooner than isogeneic petites. We conclude that (1) maintenance and expression of the virus at elevated temperatures require a combination of host and viral factors and (2) the existence of naturally-occurring ts variants may facilitate identification of specific ts factors involved in virus replication.

Host and Viral Factors Affect the Heat Curability of Killer Virus of Yeast†. L. WEINSTEIN*, F. CAPALDO-KIMBALL, M.J. LEIBOWITZ, D. ABEL, S. ROSBOROUGH, E. LAING, UMDNJ-Robert Wood Johnson Medical School, Piscataway, N.J.

Killer virus is a cytoplasmically-inherited virus of Saccharomyces cerevisiae. Its genome consists of two separately encapsidated segments of double-stranded (ds) RNA (M and L-A). The different strains of killer virus are denoted K1, K2, etc. We are studying the heat-induced loss of killer virus from various host-virus combinations to optimize a temperature-sensitive (ts) model for studying the replication and expression of dsRNA viruses in eukaryotic hosts. Virus-infected cells were grown for 100 hours at 37°C. Curing was tested at varying times by killer phenotype assays on petri dishes, and by polyacrylamide gel electrophoretic analysis of dsRNA. We have determined that (1) virions containing the L-A dsRNA segment from K1 strains (L-A-HN) are more curable than virions containing K2 type L-A dsRNA (L-A-H), (2) viruses of either type are cured more readily in diploid than in haploid cells, and (3) grande yeast cells harboring the virus are cured of the virus sooner than isogeneic petites. We conclude that (1) maintenance and expression of the virus at elevated temperatures require a combination of host and viral factors and (2) the existence of naturally-occurring ts variants may facilitate identification of specific ts factors involved in virus replication.

GENETICS OF HEAT CURABILITY OF THE KILLER VIRUS OF *SACCHAROMYCES CEREVISIAE*; *IN VITRO* MUTAGENESIS AND PHENOTYPE TESTING OF CDNA CLONES. Michael J. Leibowitz, Denise E. Georgopoulos, and Lee Ann Weinstein.
Dept. of Molecular Genetics and Microbiology, UMDNJ-R. W. Johnson Medical School, 675 Hoes Lane, Piscataway, NJ 08854-5635, U.S.A.

Yeast strains carrying the K_1 and K_2 killer traits differ in their susceptibility to heat curing, in that K_1 strains are generally more curable. All killer strains previously tested for curability have been either K_1 killers harboring M_1 and L-A-HN or M_2 and L-A-H viral dsRNA segments. We have isolated isogenic yeasts harboring recombinant viruses: M_2 L-A-HN by crossing a cryptopleurine-cured K_1 strain with a K_2 strain and M_1 L-A-H by crossing K_1 and K_2 strains at 37°C. When the four isogenic diploid recombinant and wild-type killers were tested for heat curability, this trait congregated with the L-A rather than the M dsRNA, i.e., L-A-HN strains of either killer specificity are more curable than L-A-H strains. Since L-A dsRNA encodes the major capsid protein and a capsid-RNA polymerase fusion protein, it appears that the L-A-HN dsRNA is a naturally occurring viral mutant temperature-sensitive in an unidentified step in viral replication.

The toxin-encoding M_1 dsRNA segment is transcribed *in vitro* by virus-associated RNA polymerase to yield full-length transcripts having apparent hairpin structures at their 5′ termini, which include the putative initiating AUG codon and nearby sites of complementarity to 18S and 5.8S rRNA molecules. We have constructed a full-length cDNA clone of M_1 dsRNA by linking cDNA for preprotoxin (M_1-1 region, provided by D. Y. Thomas) with M_1-2 cDNA generated in this lab. This clone contains a DNA linker replacing the 200 base polyA·polyU tract in the dsRNA, but otherwise is a complete copy. Synthetic primers colinear with the 5′ terminus of the (-) strand and containing the sequence of the 5′ terminus of the (+) strand linked to a bacteriophage SP6 promoter have been used in a polymerase chain reaction (PCR) to yield cDNA consisting of the M-derived sequences and the 17 bp SP6 promoter, with no other extraneous sequence. This PCR product was used to generate authentic m transcript *in vitro* in a reaction catalyzed by SP6 RNA polymerase; the sequence of the 5′ end of this transcript has been confirmed by RNA sequence analysis. This m transcript can be produced in the presence or absence of the preformed cap precursor m7GpppG; the transcript produced in the presence of cap is 10 times as active as a translational template in the yeast cell-free translation system. By utilizing (+) primer with altered sequence, mutant PCR products were generated which were used to generated mutant capped and uncapped RNA transcripts, whose 5′ sequence was determined. Mutations in the 5′ region of m transcript alter its activity as a translational template. However, different mutations do not have the same effect on the translatability of capped and uncapped transcript. This suggests that translational initiation on the apparently uncapped viral messages produced *in vivo* may occur by a mechanism distinct from that of capped nuclear messenger RNA molecules. The structural requirements for translation of capped and uncapped messages in yeast are being compared using this method of *in vitro* mutagenesis and phenotype testing.